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Strain-Dependent Alterations in the Expression of Folate Pathway Genes Following Teratogenic Exposure to Valproic Acid in a Mouse Model

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The molecular basis for the well-established hierarchy of susceptibility to valproic acid-induced neural tube defects in inbred mouse strains was examined using in situ transcription and anti-sense RNA amplification methodologies with both univariate and multivariate analyses of the resulting gene expression data. The highly sensitive SWV strain demonstrated a significant reduction in the expression of the folate binding protein (*FBP-1*) following the teratogenic insult at gestational day 8:18, while the more resistant LM/Bc embryos were upregulating this gene in response to valproic acid treatment. More importantly, at all 3 gestational timepoints spanning the period of murine neural tube closure examined in this study, the LM/Bc embryos had significantly higher *MTHFR* (5,10-methylenetetrahydrofolate reductase) gene expression levels compared to the SWV embryos. As this folate pathway enzyme is important in homocysteine and methionine metabolism, it suggests that the SWV embryos may be hypomethylated, and essential gene expression during critical periods of neural tube closure is compromised by the teratogenic exposure to valproic acid. This study represents the first evidence of a strain difference in transcriptional activity in response to a teratogenic exposure that might be causally related to the development of the teratogen-induced congenital malformations. Am. J. Med. Genet. 70:303–311, 1997.

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INTRODUCTION

The presence of a maternal seizure disorder and subsequent antiepileptic drug therapy has long been recognized as a major pregnancy risk factor for congenital malformations [Janz, 1975; Dansky and Finnell, 1991; Finnell et al., 1995]. Over the past three decades, a substantial body of experimental evidence has accumulated which suggests that the basis for the increased malformation risk lies not in the maternal convulsive disorder proper, but in the teratogenicity of the anticonvulsant drugs [for review see Dansky and Finnell, 1991; Finnell et al., 1995]. Most clinical and epidemiologic studies show that the incidence of congenital malformations among the offspring of antiepileptic drug-treated women with seizure disorders is approximately 2–3 times the rate observed in either nontreated epileptics, or that of the general population [Finnell et al., 1995]. The nature of the adverse effects of antiepileptic drugs on developing fetuses has been well documented in these human clinical and epidemiologic studies, and many of the clinical observations have been faithfully replicated in experimental animals [Finnell, 1981; Finnell et al., 1988, 1989; for review see Finnell and Dansky, 1991].

Together with carbamazepine (Tegretol®, Ciba-Geigy, New York), valproic acid (Depakene®, Abbott Laboratories, No. Chicago, IL) has been shown to be associated with an increased risk for posterior neural tube defects [Robert, 1982; Robert and Giubaud, 1982; Lammer et al., 1987; Rosa, 1991; Lindhout et al., 1992]. It has been estimated that 2% of all infants exposed to valproic acid during early pregnancy will have spina bifida, a 20-fold increased prevalence over that observed in the general population [Lammer et al., 1987]. In addition to neural tube defects, a characteristic pattern of craniofacial anomalies has also been described among infants exposed in utero to valproic acid. The affected children have flat nasal bridges with upturned nasal tips, thin vermilion borders, a shallow philtrum, and

downturning mouths [DiLiberti et al., 1984; Ardinger et al., 1988]. Along with congenital malformations, there is an increased risk for perinatal distress, low Apgar scores, postnatal growth deficiency, and microcephaly among valproate-exposed infants [Ardinger et al., 1988; Jaeger-Roman et al., 1986].

The mechanism by which in utero valproic acid exposure actually alters normal morphogenesis remains unknown. One potential mechanism by which this compound may exert its teratogenic effects is via altered folate metabolism. There is strong clinical and experimental evidence that periconceptional folate supplementation reduces the risk for neural tube defect-affected pregnancies [Smithells et al., 1983; Czeizel and Dudás, 1992; Shaw et al., 1994; Werler et al., 1993]. Furthermore, animal studies have shown a significant reduction in the frequency of neural tube defects in Han:NMRI inbred mice when folinic acid was coadministered with teratogenic concentrations of valproic acid [Trotz et al., 1987; Wegner and Nau, 1991, 1992]. Thus, there appears to be a clear linkage between valproic acid-induced neural tube defects and folic acid utilization.

Given the relationship between valproic acid exposure and neural tube defects in humans, and the well-established differences in susceptibility to valproic acid-induced neural tube defects in highly inbred mouse strains [Finnell et al., 1988], we were interested in examining potential differences in gene expression that might explain the observed differences in embryonic sensitivity. We selected the folate binding protein genes (*FBP-1* and *FBP-2*) for examination as they are critical for folate binding and transport into the cytoplasm of the cell. The structural genes coding for the folate receptors have only recently been cloned and sequenced [Brigle et al., 1991]. Full-length cDNAs consist of a 765 bp open reading frame for *FBP-1,* the gene believed to be homologous to the human folate receptor alpha (FRα), and a 735 bp open reading frame for *FBP-2,* the homologue of the human *FR*-β. The putative signal sequences of the two FBPs are dissimilar in residue composition and predicted charge, which may suggest targeting of these proteins to different cellular compartments or membrane types [Brigle et al., 1991]. In fact, *FBP-2* has a greater homology with the human *FR*-β than it does with *FBP-1* [Brigle et al., 1991]. Functional comparisons of the mouse *FBP-1* and *FBP-2* proteins in erythroleukemia cell lines have indicated marked differences in the binding affinity of *FBP-2* and *FBP-1* for natural folates, as well as for folate analogues, with *FBP-1* showing a 10–90-fold higher affinity for both types of compounds [Brigle et al., 1994a,b]. Since most embryonic cells lack folate receptors [Page et al., 1993], the presence in high concentrations of the receptors on the maternal placenta [Weitman et al., 1992] as well as on the syncytiotrophoblast and fetal neuroepithelium, is highly suggestive of a critical role for folate in the normal morphogenetic events involved in neural tube closure. We additionally examined the expression of the *MTHFR* gene, which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-CH_3-THF), and is important for regulating serum homocysteine. A specific polymorphism reported in this gene has been suggested as a risk factor for neural tube defects [van der Put et al., 1995; de Franchis et al., 1995; Ou et al., 1996]. These 3 genes, all extremely important for embryonic folate transport and utilization, represent excellent candidate genes for investigations into the mechanisms underlying valproic acid-induced neural tube defects.

MATERIALS AND METHODS

Experimental Animals

The highly inbred SWV and LM/Bc mouse strains were selected for these studies on the basis of their known differences in sensitivity to valproic acid-induced neural tube defects [Finnell et al., 1988]. The mice were maintained on a 12-hour light cycle in the Laboratory Animal Resources and Research Facility at the College of Veterinary Medicine, Texas A&M University. The animals were pathogen free with no apparent health problems. Up to 5 healthy females were housed per polycarbonate cage, and were allowed free access to Wayne TekLad rodent chow and tap water. Virgin females, 40–60 days of age, were bred overnight to experienced males. Females were examined the next morning for the presence of vaginal plugs. The beginning of gestation (day 0) was set at 10 P.M. of the previous evening, the midpoint of the dark cycle [Snell et al., 1948].

Teratogen Administration

For the teratology studies, no less than 10 dams were randomly assigned to each treatment group and exposed to a single intraperitoneal injection of valproic acid (600 mg/kg) or the distilled water vehicle. The drug was dissolved in distilled water immediately prior to use and administered in volumes of 0.1 ml/10 g body weight. Following administration, the dams were returned to their home cages until the desired gestational day for the embryo collection. At the assigned hour (gestational days 8.5, 9.0, 9.5, and 15.5), pregnant dams were killed by cervical dislocation, the abdomen opened, and the uterine contents removed. The location of all viable embryos or fetuses and resorption sites were recorded. Using watchmaker's forceps, the embryos were dissected free of the decidual capsule and its chorion and amnion while in cold phosphate-buffered saline, under a Wild M8 dissecting microscope (Heerbrugg, Switzerland). The embryos were grossly examined morphologically with special attention paid to the extent of neural tube closure for the embryos, and for the presence or absence of exencephaly in the gestational day 15.5 fetuses.

Removal of Neural Tube From NTC Stage Embryos

For the gene expression studies, no less than 3 embryos per litter from a minimum of 6 litters per treatment group were used as the basis of the dataset. The embryos were collected as described above. With the aid of watchmaker's forceps, the neural tube proper was dissected away from supporting paramesodermal tissue under the dissecting microscope [Taylor et al.,

1995; Wlodarczyk et al., 1996]. Once dissected free, it was examined to ensure that only the intact neural tube tissue had been collected, free from extraneous tissues. In preparation for the in situ studies, this tissue was placed in a hybridization buffer containing 5 mM DTT, 100 U RNasin (Promega, Madison, WI), and 0.1% digitonin. Following a brief pulse with a sonic dismembranator, an additional 50 U of RNasin was added to the buffer and the tissue was frozen at –80°C until further processed.

In Situ Transcription and aRNA Amplification

In situ transcription and anti-sense RNA amplification (RT/aRNA) procedures were performed according to the methods described by Eberwine et al. [1992a,b; Taylor et al., 1995; Wlodarczyk et al., 1996]. The initial steps involved making an RNA/DNA hybrid molecule from the population of mRNAs present in each of the isolated neural tube samples with the addition of avian myeloblast reverse transcriptase (Seikagaku America Inc., Bethesda, MD), and an oligo-dT-T7 oligonucleotide primer that hybridizes to the poly-A tail of the mRNAs. The resulting single-stranded DNA was isolated and allowed to form double-stranded complementary DNA (cDNA) by hairpin loop formation. The hairpin loop was digested with S1 nuclease. This double-stranded cDNA was used to produce radiolabeled, amplified, aRNA by the addition of T7 RNA polymerase (Epicentre Technologies, Madison, WI) in the presence of [^{32}P]-CTP. The aRNA, representing the entire population of mRNAs from the neural tube tissue, was then hybridized to "reverse" Northern blots in order to quantitatively determine the pattern of gene expression for the candidate genes selected for investigation. This approach was selected over more conventional reverse transcription-polymerase chain reaction (RT-PCR) procedures, given the linear nature of the RT/aRNA amplifications. The aRNA, after a single round of amplification, is increased several thousand fold, and has a high degree of fidelity to the original mRNA population in the embryo, without a great deal of skewing to give disproportionately high amounts of selected messages [Eberwine et al., 1992a]. The degree of skewing has been shown to be orders of magnitude greater in PCR amplifications when compared with the aRNA amplifications. This is a very important consideration for our studies, as we desire a highly accurate depiction of the total RNA population in the proportions present in the original embryonic tissue. The potential for high-abundance messages being disproportionately amplified by PCR was another reason why the RT/aRNA methodology was utilized in these studies.

Genetic Expression Profiling

Equimolar concentrations of the cDNA clones of the 3 genes of interest (*FBP-1, FBP-2,* and *MTHFR*) were immobilized on a nylon membrane (Zetaprobe, BioRad, Richmond, CA) along with an internal control cDNA (cyclophilin), using a BioRad slotting apparatus following the manufacturer's protocol. The resulting slot blots were prehybridized with an aRNA probe using conditions detailed previously by Taylor et al. [1995]. Briefly, each blot was prehybridized for 30 minutes in buffer containing 7% SDS (w/v), 0.12 M Na_2HPO_4(pH 7.2), 0.25 M NaCl, and 50% formamide at 42°C. The heat-denatured aRNA probes were applied to the blots and hybridized for 24 hours. At this point, the slot blots were washed at medium stringency down to 0.1 × SSC containing 0.1% SDS at 42°C, dried, wrapped in plastic wrap, and placed in an Ambis 101 2-dimensional radioanalytics imaging detector (Scanalytics, Billerica, MA) which directly measured the radioactivity (CPMs) of each slot on the reverse Northern blots. The individual signals were normalized to cyclophilin gene expression. The selection of cyclophilin as the normalizing cDNA enabled us to make comparisons between different blots, as the individual hybridization intensities of each cDNA on each blot could be expressed as a ratio of its expression to cyclophilin. There was no significance to the selection of this gene, other than the fact that it is a constitutively expressed gene found in high abundance in the mouse brain, thymus, and embryo [Danielson et al., 1988], and may be the best internal standard for use in multiprobe assays when examining low-abundance messages.

Statistical Analysis

The fetal data were subjected to univariate and multivariate statistical procedures. All statistical computations were performed by Statistical Analysis Systems [SAS Institute, 1990]. Included in the univariate analyses was a frequency comparison of resorptions and neural tube defects, which was performed using the TTEST procedure. Other univariate procedures involved determination of statistical significance ($P < 0.05$) in the gene expression profiling studies, both within and between strains. Due to the factorial nature of the treatment combinations, the testing of these experiments required investigation of the interactive effects of treatment and strain. Therefore, tests to determine treatment differences within each strain followed by tests to determine treatment differences across strains were conducted. All strain and treatment interaction comparisons were evaluated by analysis of variance (ANOVA) and the least square means (LSMEANS) option in the general linear models (GLM) procedure. The LSMEANS option computed model-based estimates of arithmetic means when there were unequal sizes of the experimental groups. Therefore, GLM was used to determine the level of significance for means among treatment classes, while adjusting for unbalanced sample sizes. For comparisons within each strain, LSMEANS was used to calculate the adjusted means for each control and valproic acid-treated experimental group, and contrasted these treatment means separately for each time point. To examine strain differences in terms of transcriptional activity, the LSMEANS procedure calculated the adjusted means of the differences between the control and the teratogen-treated means separately for each time point and strain. This procedure then contrasted these mean differences (representing a specific treatment, time-point and strain) to test the hypothesis that the effect of valproic acid treatment on folate gene expression is

consistent across strains. Statistical significance for all of the univariate analyses was set at the alpha 0.05 ($P < 0.05$) level [Sokal and Rohlf, 1981]. For a description of the least square estimation formula, see Neter et al. [1989], and for a summary of the LSMEANS option see SAS Institute [1990]. In order to avoid confusion, the adjusted means computed by LSMEANS in these analyses will be referred to throughout the remainder of the text as "means."

Two exploratory multivariate procedures, principal components analysis (PCA) and discriminant function analysis (DFA), were performed to examine the coordinate interaction of the 3 folate genes, and to better understand their coordinated influence during neural tube development. PCA, a data dimension-reduction technique, was used to mathematically consolidate the original data on the expression levels of the 3 genes into new variables known as principal components (PCs). These PCs, which represented all 3 folate genes, allowed for simultaneous examination of expression values for these genes. The PC computations were performed by a series of mathematical procedures previously described [Rao, 1973; Rohlf and Bookstein, 1990; Johnson and Wichern, 1992]. Each PC possessed a set of values, or eigenvectors, that represented linear combinations of the set of original variable values. The maximum number of PCs generated was equal to the number of original variables, such that each PC accounted for some proportion of the total variance. This proportion was represented by eigenvalues of the covariance matrix [Prakash and Murty, 1995]. The major, or first PC, possessed a maximum variance, while subsequent PCs retained increasingly smaller variances. The first 2 PCs represented the majority of the total variation associated with the original variables and were thus, sufficient to summarize the data. A 2-dimensional plot displaying the embryos on the PC axes provided an informative way to visualize the interactive roles of the folate genes on the distribution and characterization of the embryo groups (Fig. 1). Such a visualization served to reveal relationships not previously suspected by the use of simple univariate comparisons between the paired variables.

DFA was utilized to explore differentiating features of the embryo treatment groups in terms of folate gene expression. Briefly, this procedure derived canonical variables, or linear combinations of the quantitative variables, that summarized the variation achieved between embryo groups in a manner similar to the summarization of total variation by PCA. Plots of the canonical variables generated by the DFA were utilized to visualize the segregation of the treatment groups, based on the developed classification criteria. The classification criterion was derived from the pooled covariance matrices, yielded a linear discriminant function, the computations of which were described by Johnson and Wichern [1992] and Rao [1973].

For the present study, PCA was utilized to describe the simultaneous impact of folate gene expression variability on control and valproic acid-treated embryos within each strain. DFA was utilized to explain treatment group and strain differences as a function of the within-group gene expression patterns determined by the PCA.

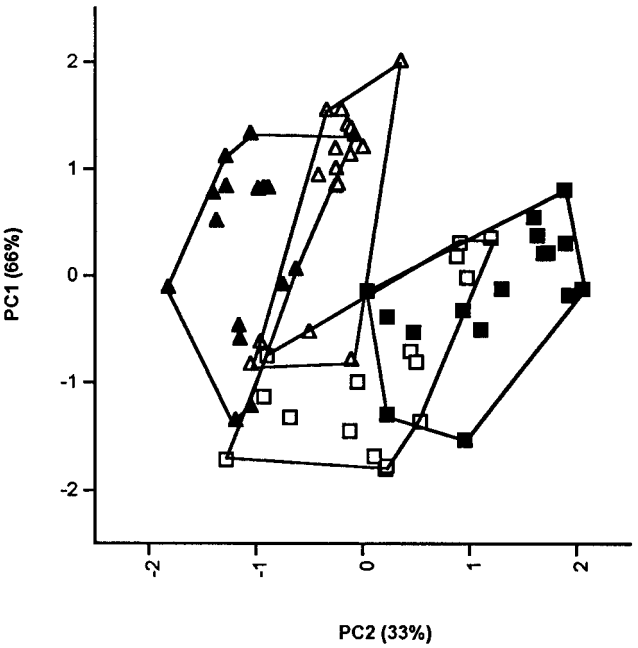

Fig. 1. PC plot of LM/Bc and SWV embryos from gestational day 8:18 in the new coordinate space defined by PCs 1 and 2 utilizing expression data for 3 folate pathway genes. The encircled clusters represent embryos from similar treatment groups. △, SWV control; ▲, SWV treated; □, LM/Bc control; ■, LM/Bc treated.

RESULTS

Teratology Results

The results of a single, intraperitoneal injection of valproic acid (600 mg/kg) on selected maternal and fetal parameters are detailed in Table I. Whereas there were no significant differences in the number of implanted embryos between the control and valproic acid-treated dams of either strain ($P > 0.05$), there was a significant increase in the incidence of embryotoxicity due to the valproic acid treatment in the SWV strain ($P < 0.05$). With respect to neural tube defects, the valproic acid injection on gestational day 8.5 was teratogenic in both mouse strains, with nearly 28% of the LM/Bc fetuses and 78% of the SWV fetuses having exencephaly ($P < 0.05$). The response frequency in the SWV was significantly greater than the rate observed in the LM/Bc strain ($P < 0.05$). Although not indicated in Table I, the exencephalic fetuses were observed in over 60% of the litters from both inbred mouse strains following the valproic acid treatment.

Gene Expression Studies

Univariate analysis. ANOVAS were performed to compare folate gene transcriptional activity at each collection time point, both within and between the 2 inbred mouse strains. This was accomplished by first calculating and comparing the means between control and valproic acid treatment groups for each time point and strain, calculating the means of the differences be-

TABLE I. Effect of Maternal Valproic Acid Treatment on Number of Implants, Resorptions, and Exencephalic Fetuses

Strain	Treatment (mg/kg)	Treatment day	Litters (no.)	Implants (no.)	Resorptions no. (%)	Exencephaly no. (%)
SWV	0	8.5	10	118	7 (5.9)	0 (0)
SWV	600	8.5	11	115	19 (16.5)*	75 (78.1)*
LM/Bc	0	8.5	10	89	5 (5.6)	0 (0)
LM/Bc	600	8.5	10	93	6 (6.5)	24 (27.6)*

*$P < 0.05$ from control.

tween the 2 treatment groups, and then contrasting these mean differences for significance ($P < 0.05$) across strains. Table II summarizes the results of these observations. Although the mean expression level of *FBP-1* significantly decreased ($P < 0.05$) in response to the valproic acid treatment in the SWV embryos at gestational day 8:18 compared to controls, the transcriptional activity of this gene remained significantly higher in these embryos than it was in the gestational day 8:18 LM/Bc embryos ($P < 0.05$; Table II). In addition, the mean expression level of *FBP-2* was significantly decreased in the LM/Bc embryos exposed to valproic acid at GD 9:12 compared to controls ($P < 0.05$). The transcriptional activity of this gene remained significantly higher in the LM/Bc embryos compared to the level of expression observed in the SWV embryos ($P < 0.05$). Furthermore, the mean expression levels of the *MTHFR* gene was significantly increased in response to valproic acid treatment in the LM/Bc embryos at all three collection time points, compared to controls ($P < 0.05$; Table II). The transcriptional activity of *MTHFR* remained significantly higher among the LM/Bc embryos at each collection time point, compared to the SWV embryos ($P < 0.05$).

Multivariate statistics. A PCA was performed on the *FBP-1, FBP-2,* and *MTHFR* expression data generated from 32 LM/Bc and 32 SWV/Fnn embryos collected at gestational day 8:18 under control and valproic acid-treated conditions. Each strain was represented by 16 embryos from each of the 2 experimental groups. This analysis was performed in an effort to define correlative gene expression variability within strains for each treatment group. The gestational day 8:18 timepoint was selected as an analytical focus because it directly precedes the onset of closure II in the SWV embryos, and therefore may reflect critical genetic differences among genes related to the growth and development of the neural tube. Following the PCA, a DFA was performed on the SWV embryos from gestational day 8:18, using only the *FBP-1* and *MTHFR* genes to explain the treatment group and strain differences as a function of the within-group gene patterns. The rationale for focusing on the SWV embryos for the DFA was based on their strain-specific susceptibility to neural tube defects in response to valproic acid. The 2 genes, *FBP-1* and *MTHFR,* were chosen for the DFA on the basis of their statistical significance in the univariate analyses, and the influence of *MTHFR* on the PCA at gestational day 8:18.

Principal components analysis. For the PCA, the 3 folate genes were reduced to 2 PCs, which accounted for approximately 66% and 33% of the total variation, respectively. The PC plot in Figure 1 illustrates the relationships between treatment groups for each strain, while Table III summarizes the eigenvector values. Although some overlapping of groups occurred, this plot discloses distinct separations not only between the strains, but also between the treatment groups within each strain along PC1 and PC2. The main separation between strains is seen along a 45° angle involving both PCs, whereas the separations between treatment groups within each strain are primarily restricted to PC2. The high eigenvector values for *FBP-1* and *FBP-2* on PC1, and that of *MTHFR* on PC2, indicate that the 45° strain separation along PCs 1 and 2 is related to the coordinate expression of these genes. The graphical location and distribution of the embryo clusters reflect the expression patterns of these 3 genes. For example, the positive eigenvector values of the FBPs on PC1 (Table III) are indicative of higher expression levels of these genes among embryos situated in the positive realm of the graph along the PC1 axis. Therefore, among the SWV embryos, the expression levels of the FBP genes are covarying at a higher degree than they are among the LM/Bc embryos. In

TABLE II. Least Square Mean Response of Folate Genes

		Gestational day					
		8:18 Treatment		9:0 Treatment		9:12 Treatment	
Strain	Genes	Control	VPA	Control	VPA	Control	VPA
SWV	*FBP-1*	22.7	16.2*,**	10.0	11.7	29.0	26.2
SWV	*FBP-2*	44.1	34.5	19.5	22.9	60.3	51.5
SWV	*MTHFR*	2.7	1.4	1.6	1.9	3.3	2.7
LM/Bc	*FBP-1*	9.2	14.0	9.9	12.7	21.4	17.4
LM/Bc	*FBP-2*	18.5	28.9	21.1	26.1	46.1	35.2*,**
LM/Bc	*MTHFR*	6.0	15.3*,**	1.9	6.9*,**	1.8	6.0*,**

*$P < 0.05$ from controls within strains.
**$P < 0.05$ from controls between strains.

TABLE III. Eigenvector Values for the Influential PCs*

	Eigenvectors	
Genes	PC1	PC2
FBP-1	**0.7025**	0.0930
FBP-2	**0.7048**	0.0461
MTHFR	−0.0984	**0.9945**

*Bolded values signify heavily weighted genes. PCA is based on the expression values of the 3 folate genes.

contrast, the LM/Bc embryos are expressing higher levels of *MTHFR* relative to the SWV embryos. Of particular importance in this analysis was the graphical distribution of the embryos within treatment groups for each strain. The SWV control group had a fairly wide range of FBP gene expression among the embryos, which was suggested by its distribution along PC1. However, the distribution was quite narrow along PC2, indicating a potentially narrow tolerance range for alterations in *MTHFR* expression levels among SWV embryos. In addition, the shift in cluster position to the left of the graph, following treatment in the SWV embryos, may indicate an influence of decreased *MTHFR* expression levels on the tolerance of these embryos to *MTHFR* fluctuations. That is, the valproic acid-treated SWV embryos that fell outside of the distribution defined by the control group were characterized by their decreased *MTHFR* gene expression levels, and may be at high risk for a neural tube defect. Among the LM/Bc embryos, there was a shift along PC2, following the teratogenic insult towards a higher level of *MTHFR* expression, relative to that observed among the control embryos (Fig. 1). However, the distribution within each group remained visibly consistent among the LM/Bc embryos. The consistency in the embryo distribution range may indicate a higher resiliency to fluctuations in folate gene expression levels than that observed in the SWV embryos. Furthermore, the shift in position along the PC2 axis may indicate a protective effect by the embryos to increase transcription of *MTHFR* in response to a teratogenic valproic acid exposure.

Discriminant function analysis. A linear DFA was performed to construct the classification criterion, using all 32 SWV embryos from gestational day 8:18, and the expression values representing the *FBP-1* and *MTHFR* genes. This procedure focused exclusively upon the SWV strain, in an attempt to explain the gene expression differences between the control and valproic acid treatment groups as a function of the PCA results. Four embryos (12.5%) were misclassified into erroneous treatment groups: 2 control embryos were misclassified as being valproic acid treated, whereas 2 valproic acid-treated embryos were misclassified as being from the control sample. These low misclassification rates indicate a high degree of consistency among treatment groups for this classification scheme, suggestive of a successful DFA. The canonical variables plot in Figure 2 illustrates the segregation of SWV control and teratogen-treated embryos in this analysis.

DISCUSSION

This study represents the first investigation at a molecular level to determine the basis of the difference in susceptibility to valproic acid-induced neural tube defects observed among inbred mouse strains. While the highly sensitive SWV fetuses had exencephaly response frequencies approaching 80% affected when the dams received a single intraperitoneal injection of valproic acid, less than 30% of the LM/Bc fetuses were affected following an identical valproic acid exposure. This large difference in the response frequencies to valproic acid-induced exencephaly in the 2 mouse strains suggests that susceptibility to these malformations has a strong genetic component [Finnell et al., 1986, 1988; Finnell, 1991; Seller et al., 1979]. Given the abundance of highly suggestive clinical and experimental literature published during the last few years linking folates and neural tube defects, we focused our initial efforts to explain the strain differences on a few genes involved in folate transport and metabolism.

With respect to folic acid metabolism, cells acquire their necessary folate in the form of 5-CH_3-THF from the bloodstream by a strictly regulated, high affinity uptake process known as potocytosis [Anderson et al., 1992]. During potocytosis, 5-CH_3-THF is bound to its specific extracellular receptor which is anchored to the plasma membrane via a glycosyl-phosphatidylinositol linkage [Rothberg, 1990]. Once these receptors are saturated, the cell membrane invaginates creating a nonclathrin-coated vesicle called a caveolae, which remains attached to the internal surface of the membrane. 5-CH_3-THF is released from its receptor via an acidification of the caveolae, and is then delivered to the cytoplasm of the cell by a specific carrier. The murine genes which appear to be responsible for the regulation of the binding and transport of 5-CH_3-THF into

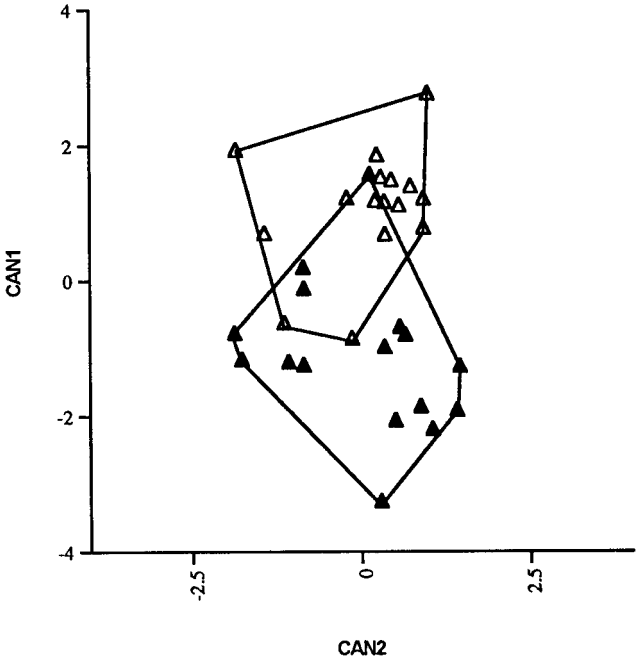

Fig. 2. Discriminant function plot of SWV embryos from gestational day 8:18 on the first and second canonical variates, utilizing expression data for 2 folate pathway genes determined to be influential in the first PCA and the univariate analysis. The encircled clusters represent embryos from similar treatment groups. △, SWV control; ▲, SWV treated.

the cell are the folate binding protein genes, *FBP-1* and *FBP-2* [Brigle et al., 1991, 1994a,b]. Dysfunctional receptors result in lower intracellular 5-CH_3-THF concentrations that can be stoichiometrically driven upwards by exogenously administered folate. Of all the steps involved in potocytosis, however, the binding of 5-CH_3-THF to the receptor is the only known folate-specific step [Kamen et al., 1988], making the folate binding proteins excellent candidate genes for determining susceptibility to neural tube defects.

Previously published animal studies provide additional support for the potential connection between the folate biosynthetic pathway and valproic acid-induced neural tube defects. Wegner and Nau [1991, 1992] measured the levels of total folate and several folate metabolites in mice following teratogenic exposure to valproic acid. They found that while the total folate concentrations in both the dam and embryos remained constant in response to the valproic acid treatment, there was a significant alteration in the concentrations of several formylated tetrahydrofolates. Specifically, valproic acid reduced the concentrations of the 5 and 10-formyl-THF, as well as the 5-CH_3-THF metabolites. This decrease in the formylated THFs could be the result of a valproic acid-induced inhibition in the interconversion of THF and formylated metabolites by glutamate formyltransferase [Wegner and Nau, 1991, 1992]. When these metabolites were measured in valproic acid-exposed embryos from the neural tube defect-sensitive (SWV) or resistant (DBA/2J) strains, there were significant differences in their metabolic profiles. Specifically, there was between an 86% and 92% inhibition of the 5-CHO-THF and 5-CH_3-THF metabolites in the SWV embryos, whereas the DBA/2J embryos had no alterations in their 5-CH_3-THF, and only a 50% inhibition in the 5-CHO-THF metabolite. The LM/Bc strain embryos were intermediate between the SWV and DBA/2J embryos in both susceptibility to valproic acid-induced neural tube defects and their profile of metabolic inhibition [Finnell et al., 1995]. This reduction in specific folate metabolites in the SWV embryos may adversely affect purine biosynthesis, which could have significant consequences to the embryo's ability to synthesize DNA as well as downstream expression of essential genes during critical periods of embryogenesis.

The processes involved in neural tube closure are controlled by a complex, coordinate network of gene expression within the developing neuroepithelium and supporting mesenchymal cells. It is highly regulated both spatially and temporally, so that even minor alterations in gene expression have the potential to significantly disrupt the coordinated events of neurulation. At gestational day 8:18, cells of the neural plate are actively involved in proliferation and the formation of the neural folds. This is a critical period for the proper development of the neural tube. The gene expression data obtained from the neural tubes of the 2 inbred strains examined in these studies clearly demonstrate a difference in the transcriptional activity of the folate genes. Specifically, the sensitive SWV strain had a very high rate of *FBP-1* expression, which was significantly downregulated at this timepoint in response to the valproic acid treatment (Table II). The high level of expression observed in the SWV control embryos implies that the gene is critical to the developing neural tube at this timepoint, as DNA synthetic activity increases to meet the growth demands of the embryo. The resulting downregulation of its expression may be causally related to the high rate of neural tube closure defects following the valproic acid exposure. In the more resistant LM/Bc strain, *FBP-1* expression at gestational day 8:18 actually increases following the valproic acid treatment (Table II) to levels which were similar to those observed in the SWV embryos. Previously, our laboratory has shown that the SWV embryos lag behind the LM/Bc in their progression through neural tube closure [Finnell et al., 1993; Wlodarczyk et al., 1996; Finnell and Bennett, unpublished]. Therefore, it is quite possible that by gestational day 8:18, the LM/Bc embryos had progressed to a stage of neural tube development which was immune to the slight perturbations in folate transport gene expression induced by valproic acid treatment.

In the mouse, *FBP-1* is considered to be homologous to the human *FR*α, whereas *FBP-2* is the homologue of human *FR*β. As such, we would expect *FBP-1* to have the primary role in murine folate transport. Functional comparisons of *FBP-1* and *FBP-2* proteins in erythroleukemia cell lines have indicated marked differences in the binding affinity of these receptors for natural folates, as well as folate analogues, with *FBP-1* showing a 10–90-fold higher affinity for both types of compounds [Brigle et al., 1994a,b]. Based upon our gene expression data, it certainly appears that *FBP-1* is the more important of the 2 genes with respect to potential involvement in the underlying mechanisms of valproic acid-induced neural tube defects.

At all 3 embryo collection timepoints examined in these studies, there was a significantly different pattern of *MTHFR* gene expression observed between the 2 mouse strains. The level of expression for this gene did not change from control levels in the sensitive SWV strain in response to the valproic acid treatment (Table II). In the LM/Bc strain, *MTHFR* expression was significantly increased following valproic acid treatment over levels observed in the controls, or in the valproic acid-treated SWV embryos (Table II). These results were substantiated by the PCA analysis, in which *MTHFR* dominated the expression pattern of the LM/Bc mice in general, especially among valproic acid-treated embryos. This is shown in Figure 1 by the dramatic shift in the graphical location of the valproic acid-treated LM/Bc embryo cluster along PC2. Also noteworthy were the shapes of the embryo distributions, which show that the coordinated expression patterns of *MTHFR, FBP-1,* and *FBP-2* are widely divergent between strains, but relatively consistent between the treatment groups within the LM/Bc embryos. The difference between the SWV treatment group distributions was primarily due to an alteration in the proportional expression of *MTHFR* relative to that of the FBPs in response to teratogenic concentrations of valproic acid. The results of the PCA are substantiated by the DFA. The high level of *MTHFR* gene expression in the LM/Bc embryos in response to the teratogenic chal-

lenge may serve to protect the embryo by enhancing the level of DNA methylation of selected downstream transcription and growth factors. Whereas these target genes might be hypomethylated due to the constitutive or static expression of *MTHFR* in the SWV embryos. This lack of essential gene expression in the SWV embryos during critical periods of neural tube closure may result in the development of the neural tube defect [Finnell, 1991].

The results obtained in this study support the general hypothesis that fetuses with a genetically determined susceptibility to valproic acid-induced neural tube defects have differences in their ability to transport and metabolize various products in the folic acid biosynthetic pathway that may put them at increased risk for an adverse pregnancy outcome. We have demonstrated for the first time that the strain differences in sensitivity to the induction of neural tube defects may be due to a difference in the transcriptional activity of the embryos for the *MTHFR* gene. This observation, consistent with recent clinical reports [Ou et al., 1996], suggests that alterations in the levels of methylated metabolites of THF may play a highly significant role in the susceptibility to and development of valproic acid-induced neural tube defects in the mouse. Currently, our laboratory is exploring how these alterations in the methylated folate metabolites change the expression of growth and transcription factors and determining their impact on the developing neural tube.

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